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Research Article

HIF mediated and DNA damage independent histone H2AX phosphorylation in chronic hypoxia

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Abstract

The histone variant 2AX (H2AX) is phosphorylated at Ser139 by the PI3K-like kinase family members ATM, ATR and DNA-PK. Genotoxic stress such as tumor radio- and chemotherapy is considered to be the main inducer of phosphorylated H2AX (γH2AX) which forms distinct foci at sites of DNA damage where DNA repair factors accumulate. γH2AX accumulation under severe hypoxic/anoxic (0.02% oxygen) conditions has recently been reported to follow replication fork stalling in the absence of detectable DNA damage. In this study, we found HIF dependent accumulation of γH2AX in several cancer cell lines and mouse embryonic fibroblasts exposed to physiologically relevant chronic hypoxia (0.2% oxygen) which did not induce detectable levels of DNA strand breaks. The hypoxic accumulation of γH2AX was delayed by RNAi mediated knockdown of HIF-1α or HIF-2α and further decreased when both HIF-αs were absent. Conversely, basal phosphorylation of H2AX was increased in cells with constitutively stabilized HIF-2α. These results suggest that both HIF-1 and HIF-2 are involved in γH2AX accumulation by tumor hypoxia which might increase the cancer cell's capacity to repair DNA damage, contributing to tumor therapy resistance.

Keywords: DNA damage response; oxygen sensing; tumor hypoxia.

Introduction: tumor hypoxia and therapy resistance

Hypoxia is a common feature of solid tumors and develops due to inadequate vascularization, tortuous blood vessels and high oxygen consumption. Transient blockage of red blood cell flux alternating with rapid alleviation leads to frequent periodical hypoxia/ischemia followed by reoxygenation (Yasui *et al.*, 2010). Reoxygenation, most likely mediated by the generation of reactive oxygen species (ROS), but not hypoxia can lead to detectable DNA damage (Hammond *et al.*, 2003). Hypoxia is strongly associated with malignant progression, metastatic outgrowth, genetic instability, resistance to radio-and chemotherapy and overall poor patient prognosis in various tumor types. (Brown, 1998; Brown *et al.*, 2004; Pouysségur *et al.*, 2006). Therefore, a thorough understanding of the molecular pathways in the hypoxic tumor microenvironment is warranted in order to develop new strategies for efficient cancer therapy.

Central to the cellular response to hypoxia is the heterodimeric hypoxia-inducible transcription factor HIF, consisting of one of three oxygen-labile α subunits and a common constitutive β subunit (Wenger 2002; Schofield *et al.*, 2004;). HIF activates a large number of oxygen regulated genes required for the adaptation of normal cells to hypoxia (Wenger *et al.*, 2005). In tumors, HIF-1 is responsible for the generation of new blood vessels through transcriptional regulation of the vascular endothelial growth factor (VEGF), for pH regulation by increasing the expression of carbonic anhydrase (CA) IX, and for the aerobically increased glycolytic capacity of cancer cells, also known as Warburg effect (Seagroves *et al.*, 2001; Minchenko *et al.*, 2002; Svastova *et al.*, 2004). Furthermore, hypoxic tumor cells are able to maintain metabolic functions without adequate oxygen supply via a switch to anaerobic fermentation (Pasteur effect) that is facilitated in a HIF-1 dependent manner (Schroeder *et al.*, 2005). Therefore, high HIF-1 levels in the hypoxic tumor microenvironment is a well established factor for aggressive tumor growth and a negative factor for cancer therapy (Ryan *et al.*, 1998; Ryan *et al.*, 2000; Hopfl *et al.*, 2002; Unruh *et al.*, 2003).

Besides tumor hypoxia that leads to HIF-αprotein stabilization, the loss of tumor suppressor proteins such as pVHL, p53 or PTEN or oncogenes such as v-src can contribute to high HIF-α levels in cancer cells (Jiang *et al.*, 1997; Krieg *et al.*, 2000; Ravi *et al.*, 2000; Zundel *et al.*, 2000). Both HIF-1α and HIF-2α are widely overexpressed in many human cancers and are

frequently associated with malignancy and poor prognosis (Birner *et al.*, 2000; Aebersold *et al.*, 2001). Furthermore, high HIF-1α protein levels have been shown to correlate with incomplete responses to chemotherapy and radiotherapy (Aebersold *et al.*, 2001; Koukourakis *et al.*, 2002; Bachtiary *et al.*, 2003; Generali *et al.*, 2006). Hypoxia *per se* affects radiation sensitivity since the radiation induced DNA damage is dependent on oxygen (Gray *et al.*, 1953). In addition, decreased cell proliferation and lower drug concentrations in the hypoxic tumor areas contribute to the resistance to chemotherapy. However, the underlying molecular mechanisms causing therapy resistance of hypoxic tumor cells are incompletely understood, but it is likely that HIF downstream targets are directly involved in these processes.

Targeting HIF to improve cancer therapy

HIF-1 dependent hypoxic induction of the multidrug resistance MDR1 gene was one of the first described molecular mechanism explaining the involvement of HIF-1 in chemotherapy resistance in various tumor cells, including breast carcinoma, gastric cancer, colon cancer and glioma (Comerford *et al.*, 2002; Wartenberg *et al.*, 2003; Zhou *et al.*, 2005; Nardinocchi *et al.*, 2009). Hypoxically dysregulated apoptosis in response to chemotherapy might be another explanation (Erler *et al.*, 2004; Sermeus *et al.*, 2008). The role of HIF-1 in the regulation of apoptosis is very complex and context specific. The involvement of HIF-1 in apoptosis in certain cell types cannot be generalized since cells do not undergo apoptosis under degrees of hypoxia sufficient for HIF-1 induction (Wenger *et al.*, 1998). In primary cells, hypoxia typically leads to cell cycle arrest and HIF-1 dependent apoptosis in case of more severe conditions (Greijer *et al.*, 2004). However, HIF-1 functions as a robust suppressor of apoptosis in most transformed cells. We previously reported that transformed mouse embryonic fibroblasts (MEFs) were more sensitive to chemotherapy as well as to radiotherapy in the absence of HIF-1α due to an impaired DNA double-strand break (DSB) repair capacity (Wirthner *et al.*, 2008). The underlying molecular mechanism involve markedly reduced expression of DNA-PKcs, Ku80 and Ku70, three members of the DNA-dependent protein kinases (DNA-PK), in HIF-1α deficient MEFs. Our data were supported by a large number of studies that demonstrate reversal of radio- and chemoresistance by targeting HIF-1α in various tumor types (Zhang *et al.*, 2004; Moeller *et al.*, 2005; Williams *et al.*, 2005; Brown *et al.*, 2006; Li *et al.*, 2006; Li *et al.*, 2006; Song *et al.*, 2006; Sasabe *et al.*, 2007). For example, Li *et al.* showed that knockdown of HIF-1α in

breast carcinoma cells repressed G_0/G_1 phase accumulation and relieved S phase block, thereby increasing sensitivity to chemotherapy and attenuating tumor growth (Li *et al.*, 2006). Functional interference with HIF-1α in various tumor cells has been shown to result in enhanced cell death upon treatment with chemotherapeutic agents (Ricker *et al.*, 2004; Peng *et al.*, 2006; Hao *et al.*, 2008; Sermeus *et al.*, 2008; Flamant *et al.*, 2010). On the other hand, experimentally increasing HIF-1α enhanced therapy resistance (Ji *et al.*, 2006; Martinive *et al.*, 2006). Of note, HIF-1 in germ cells of *Ceanorhabditis elegans* has recently been reported to antagonize p53-mediated apoptosis upon to DNA damage (Sendoel *et al.*, 2010).

The induction of DNA damage by cytotoxic agents has proved to be an effective strategy for cancer therapy (Einhorn, 2002; Agarwal *et al.*, 2003; Pires *et al.*, 2012). Mutations in DNA damage response (DDR) genes can lead to increased frequency and incorrect DNA damage repair, thereby contributing to genomic instability characteristic for cancer cells (Bolderson *et al.*, 2009). Because HIF-1 mediated therapy resistance was only observed when DSB but not single-strand break (SSB) inducing agents were applied, we suspected that HIF-1 might be involved specifically in DNA-DSB repair (Unruh *et al.*, 2003).

The DNA damage response in hypoxia

Upon DNA damage, histone H2AX is rapidly phosphorylated at Serine 139 by ataxia teleangiectasia mutated (ATM) kinase, ATM- and Rad3-related (ATR) kinase and DNA-PK (Fernandez-Capetillo *et al.*, 2004; Zhang *et al.*, 2006; Hurley *et al.*, 2007). Previous studies suggested that severe hypoxia can elicit a DNA damage-like response, implying the activation of the ATR and ATM pathways and subsequent phosphorylation of H2AX (Hammond *et al.*, 2003; Bencokova *et al.*, 2009). More recently, Economopoulou *et al.* identified a novel role for histone H2AX in hypoxia triggered angiogenesis (Economopoulou *et al.*, 2009). Replication specific γH2AX was found to be induced in an ATR dependent manner in endothelial cells exposed to milder hypoxia (1% O_2). Whether HIF is involved in the hypoxic induction of γH2AX has not been analyzed so far. Therefore, we investigated a potential role for HIF-1 and HIF-2 in the phosphorylation of H2AX under chronically hypoxic (0.2% O_2) conditions. Hypoxic γH2AX induction was observed in a range of cancer cell lines, was delayed in HIF-1α deficient MEFs and after HIF-1α and HIF-2α knockdown in Hek293 cells, and was further decreased when both HIFs were downregulated. *Vice versa*, in 786-0 cells, devoid of pVHL and constitutively expressing HIF-2α, H2AX phosphorylation was increased, and could be reversed by pVHL

reconstitution. These results suggest that HIF plays a crucial role in the DNA damage response under hypoxia.

Results

γH2AX accumulation in chronic hypoxia

Hammond *et al.* previously reported that severe hypoxia/anoxia (0.02% O_2) leads to ATR dependent γH2AX accumulation which was attributed to S-phase arrest (Hammond *et al.*, 2002; Hammond *et al.*, 2003). Because an atmospheric oxygen concentration of 0.02% O_2 results in a tissue partial pressure of oxygen which is most likely below the threshold for mitochondrial respiration, we investigated γH2AX induction under physiologically relevant hypoxic conditions. To ensure unimpaired mitochondrial respiration, an atmospheric oxygen concentration of 0.2% O_2 was chosen, corresponding to an oxygen partial pressure of approx. 1.5 mmHg. Ischemia-like conditions and reoxygenation induced ROS formation followed by DNA damage was prevented by replacing the cell culture medium every 24 hours with pre-equilibrated medium and by harvesting the cells inside of a hypoxic workstation. Several cancer cell lines were exposed to 0.2% O_2 for 3 to 72 hours, followed by analysis of H2AX Ser139 phosphorylation by immunoblotting. As shown in Figure 1A, γH2AX accumulated time-dependently in all six cell lines and reached maximal induction after 24 - 48 hours of hypoxic exposure, depending on the cell line. Hypoxic γH2AX induction in wild-type HEK293 cells with normal p53 was similar to SV40 large T antigen immortalized HEK293T cells, suggesting that p53 is not involved in hypoxic H2AX phosphorylation. Only wild-type HEK293 cells were used for subsequent experiments.

We next compared γH2AX accumulation in hypoxia with the effects of the topoisomerase II inhibitor and DSB inducing agent etoposide (Burden *et al.*, 1998). γH2AX slowly accumulated in hypoxia with a maximum after 48 - 72 hours and declined after 96 hours (Figure 1B). One hour of etoposide treatment with concentrations from 0.25 - 8 μM resulted in a similar, dose-dependent increase in γH2AX levels. Total H2AX levels remained unaffected after both hypoxic exposure and etoposide treatment (Figure 1B).

Hypoxic γH2AX accumulation is HIF dependent

The involvement of HIF in hypoxic H2AX phosphorylation was investigated by shRNA mediated stable knockdown of HIF-1α and/or HIF-2α in HEK293 cells. Hypoxic γH2AX accumulation was delayed after shRNA mediated knockdown of either HIF-1α or HIF-2α, with maximal levels only after 72 hours compared to 24 - 48 hours in the parental control (Figure 2A). Total H2AX remained unaffected (Figure 2A). Concomitant HIF-1α and HIF-2α double knockdown substantially decreased hypoxic phosphorylation ofα H2AX at all time points (Figure 2B).

To corroborate these findings, two different MEF cell lines derived from two different HIF-1α knockout mouse strains were analyzed. These cell lines were either only immortalized by SV40 large T (MEF-*Hif1a*$^{-/-}$T) or immortalized and transformed by H-*ras* (MEF-*Hif1a*$^{-/-}$*r*T), respectively (Feldser *et al.*, 1999; Ryan *et al.*, 2000). Importantly, these MEF cell lines were shown to lack functional HIF-2α protein (Park *et al.*, 2003). Confirming the results obtained with HEK293 cells, γH2AX levels in wt MEFs accumulated after 24 hours exposure to 0.2% O_2, but were strongly impaired in MEFs devoid of HIF-1α. Total histone levels remained unaffected as shown by Ponceau S staining of the extracted histone fraction (Figure 2C). We previously reported increased susceptibility to DNA damage with enhanced phosphorylation of H2AX in MEF-*Hif1a*$^{-/-}$*r*T upon low dose (0.5 - 4 μM) etoposide treatment (Wirthner *et al.*, 2008). However, the HIF dependent difference of γH2AX levels decreased with higher doses of etoposide and was invisible upon treatment with 8 μM (Wirthner *et al.*, 2008). In line with these findings, no HIF-1α dependent changes in γH2AX induction could be observed after high dose (8 μM) etoposide treatment which resulted in γH2AX levels that were only slightly higher than the γH2AX levels in HIF-1α positive MEFs after 24 hours of hypoxia (Figure 2C).

To further confirm the role of HIF in hypoxic γH2AX accumulation, parental and HIF-1α/HIF-2α double knockdown HEK293 cells were grown under 20% or 0.2% O_2 conditions for up to 72 hours before γH2AX levels were quantified by FACS analysis. While 88% of parental cells were strongly γH2AX positive after 48 and 72 hours of hypoxia, only 20 - 24% of the HIF-1α/HIF-2α double knockdown HEKs showed similarly elevated γH2AX staining (Figure 2D).

Finally, VHL-deficient 786-0 cells, containing constitutively stabilized HIF-2α (Maxwell *et al.*, 1999), and reconstituted 786-0-pVHL cells were cultured under 20% or 0.2% O_2 conditions for 4 - 72 hours and analyzed by immunoblotting. In line with our findings above, both basal and hypoxic levels of γH2AX were substantially higher in 786-0 cells compared to 786-0-pVHL cells (Figure 2E).

Hypoxic γH2AX accumulation is independent of DNA-DSB formation

Hypoxia has previously been suggested to induce genetic instability, associated with increased HIF-1α levels (Bristow *et al.*, 2008). However, the previously published lack of detectable DNA damage at 0.02% O_2 suggests that hypoxic γH2AX accumulation might be partially or fully independent of DNA-DSB formation (Hammond *et al.*, 2003). To directly assess DNA-SSB and DNA-DSB formation under 0.2% O_2 conditions, we performed alkaline single-cell electrophoresis (comet assays) in wild-type and HIF-1α deficient MEFs and concomitantly determined γH2AX protein levels by immunoblotting. As shown in Figure 3A, emergence of DNA-DSB induced by 1 μM etoposide could be visualized reliably by "comet halo" formation. Quantification of the median of the tail moment demonstrated a significant ($p < 0.0001$) 4-fold increase following treatment with 1 μM etoposide for 1 hour but not after up to 24 hours of 0.2% O_2 (Figure 3B, upper panel). In contrast, γH2AX levels in HIF-1α wild-type MEFs were even higher after 12 and 24 hours of hypoxia than following treatment with 1 μM etoposide (Figure 3B, lower panel). Taken together, these data suggest that DNA-DSB is not a major determinant of hypoxic γH2AX induction.

Discussion

Hypoxic regions in solid tumors result from an imbalance between cellular oxygen consumption and oxygen delivery as a consequence of inefficient tumor vasculature and limited oxygen diffusion (Chitneni *et al.*, 2011). Rapid and frequent variations in red blood cell flux cause temporal and spatial variations in the degree of hypoxia within the same tumor. We found that chronic hypoxia triggers the phosphorylation of the histone variant 2AX in a HIF dependent manner. In line with a previous report (Hammond *et al.*, 2003), we showed that γH2AX levels after chronic hypoxia were comparable with etoposide treatment. Hypoxia (0.2% O_2) did not lead to detectable DNA damage when analyzed by alkaline single cell electrophoresis. Furthermore, proliferation and cell viability were not altered, even after long

term (3 days) hypoxic exposure (data not shown). However, conditions close to anoxia have been reported to have direct cytotoxic effects and elicit apoptosis (Papandreou *et al.*, 2005). In line with our previous findings (Wirthner *et al.*, 2008), 53BP1 dose-dependently accumulated in distinct nuclear foci upon treatment with etoposide and partially overlapped with γH2AX staining (data not shown). These foci are most likely sites of DNA-DSBs. In contrast, in chronic hypoxia γH2AX did not accumulate in nuclear foci but showed a more diffuse pattern throughout the nucleus (data not shown). A similar granular γH2AX and ATM phospho-S1981 staining has been reported previously to occur in response to severe hypoxia (0.02% O_2) (Hammond *et al.*, 2003; Bencokova *et al.*, 2009). Hammond *et al.* found that severe hypoxia leads to replication fork stalling and ATR dependent γH2AX accumulation during S-phase (Hammond *et al.*, 2002; Hammond *et al.*, 2003). Moreover, diffuse and pan-nuclear γH2AX staining has been found to occur upon non-ionizing UV-C irradiation, independent of DNA-DSBs (Marti *et al.*, 2006). Infection with inactivated adeno-associated virus has been shown to lead to replication fork stalling and a diffuse γH2AX nuclear staining which was essential for subsequent cell cycle arrest in the absence of DNA damage (Fragkos *et al.*, 2009). However, the mechanism behind this diffuse γH2AX distribution pattern as well as its functional relevance are currently unknown.

DNA-DSBs are serious lesions that can lead to genomic instability if improperly repaired, or ultimately to cell death if the repair machinery is saturated. It is essential that the cell closely monitors such stress conditions and initiates signals for an adequate response. Phosphorylation of H2AX on serine 139 is established as a sensitive marker for DNA-DSBs (Bonner *et al.*, 2008). γH2AX is regarded as a key component for DNA repair, even though it seems dispensable for the initial recognition of DNA-DSBs and H2AX-deficient mice are viable (Celeste *et al.*, 2002; Celeste *et al.*, 2003).

The physiologic relevance of hypoxia induced γH2AX is poorly understood. A recent report showed that hypoxia triggered neovascularization required endothelial H2AX and γH2AX was induced in an ATR dependent manner in moderate hypoxia due to replicative stress (Economopoulou *et al.*, 2009). Genetic inactivation of H2AX was sufficient to suppress tumor angiogenesis and growth in xenograft models. However, this study did not address the question whether HIFs are involved in this effect. In the present work, we were able to show that HIF is an integral factor required for efficient phosphorylation of H2AX under physiologically relevant hypoxic conditions, and that hypoxic γH2AX induction was delayed in the absence of HIF-α. We previously reported that DNA-PK expression was strongly

reduced by the absence of HIF-1α under both normoxic and hypoxic conditions (Wirthner *et al.*, 2008), raising the possibility that DNA-PK might be the responsible kinase for H2AX phosporylation in chronic hypoxia. In line with this hypothesis, accumulation of DNA-PKcs, Ku70 and Ku80 following hypoxia and iron chelation has been demonstrated in a number of different cell lines (Ginis, *et al.*, 2000; Lynch, *et al.*, 2001; Um, *et al.*, 2004; Bouquet *et al.*, 2011). DNA-PK has been shown to phosphorylate H2AX in different cell lines and *in vivo* in response to DNA damage (Stiff *et al.*, 2004; Koike *et al.*, 2008; An *et al.*, 2010), under hypertonic conditions (Reitsema *et al.*, 2005) and during apoptotic DNA fragmentation (Mukherjee *et al.*, 2006). Of note, the hypoxic DNA-PK activation resulted in increased HIF dependent gene expression (Bouquet *et al.*, 2011). These data suggest that DNA-PK might be both upstream and downstream of HIF.

In summary, our data indicate a novel DNA-DSB independent mechanism by which HIF downstream effectors might be involved in histone H2AX phosphorylation during hypoxia and hence could contribute to therapy resistance of hypoxic cancer cells.

Materials and methods

Cell culture and lentiviral transduction

All cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, Buchs, Switzerland) as described previously (Stiehl *et al.*, 2006). For chronic hypoxic exposure, cells were grown in a gas-controlled glove box to handle the cells under constant oxygen (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK). Before medium change, all reagents were pre-equilibrated to the 0.2% O_2 containing gas mixture inside the glove box. Cell number, size and viability were determined by trypan blue exclusion using an automatic cell analyzer (Vi-Cell, Beckman-Coulter, Nyon, Switzerland). Stable knockdown of HIF-1α and HIF-2α in HEK293 cells by RNA interference was achieved by lentiviral transduction of short hairpin (shRNA) constructs. Viral particles were produced in HEK293T human enbryonic kidney cells using the ViraPower lentiviral expression system according to the manufacturer's protocol (Invitrogen, Basel, Switzerland) as described previously (Stiehl *et al.*, 2012).

Immunoblot analysis

Histone immunoblotting was performed as described previously (Wirthner *et al.*, 2008). Primary antibodies used were: γH2AX (Millipore, Zug, Switzerland); total H2AX (Millipore), β-actin (Sigma, Buchs, Switzerland). Horseraddish peroxidase coupled secondary anti-mouse and anti-rabbit antibodies were purchased from Pierce (Lausanne, Switzerland). Chemiluminescence detection was performed using Supersignal West Dura (Pierce) and signals were recorded and quantified using a charge-coupled device camera (Lightimager LAS-4000mini, Fujufilm, Dielsdorf, Switzerland). Extracted histones were stained with Ponceau S (Sigma).

Flow cytometry

Single cell suspensions were incubated with an antibody against γH2AX and propidium iodide (PI) according to manufacturer's instructions. Stained cells were analyzed with a FACSCanto II utilizing FACSDiva software (BD Biosiences, Allschwil, Switzerland).

Single cell electrophoresis (comet assays)

Alkaline single cell electrophoresis was performed as described before (Wirthner *et al.*, 2008). Briefly, MEFs were mixed with 0.5% low melting-point agarose (Sigma), solidified on microscopy slides, and lysed with 1% Triton-X100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 10.0) for 1 hour at 4°C in the dark. Horizontal electrophoresis (~0.74 V/cm; 300 mA) was performed in 300 mM NaOH, 1 mM EDTA for 30 minutes. Following SYBR green (Invitrogen) staining, DNA migration was visualized by fluorescence microscopy and the tail moment (% DNA in tail multiplied by tail length) was calculated from >150 cells per condition using the CometScore software package (TriTek, Sumerduck, VA, USA). Quantification of the median tail moments is shown as mean values ± standard error of the mean (SEM). Statistical analysis was performed applying two-tailed Student's *t*-test using GraphPad Prism version 4.0 (GraphPad Software, Ja Jolla, California, USA).

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Figure

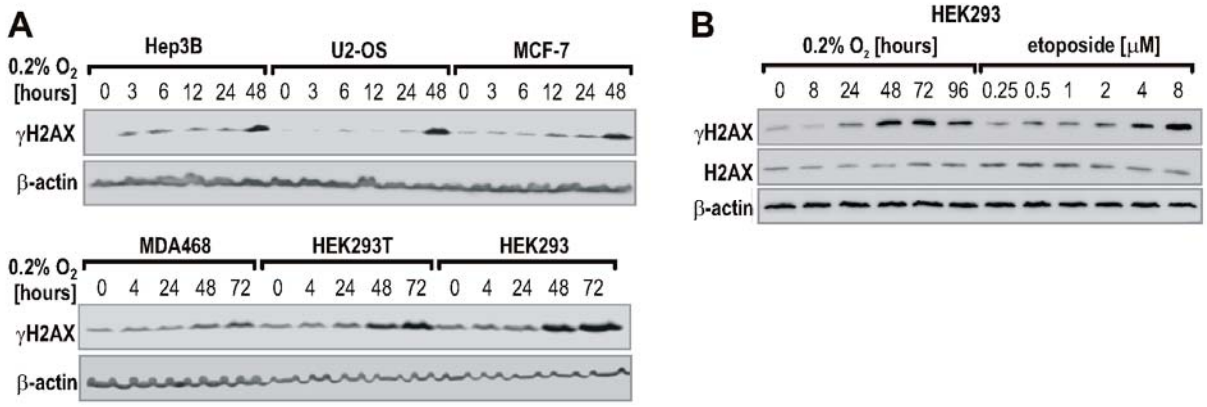

Figure 1 Phosphorylation of H2AX in chronic hypoxia.

(A) The indicated cancer cell lines were cultured under 20% or 0.2% O_2 conditions for up to 72 hours and γH2AX protein levels were analyzed by immunblotting. β-actin served as a control for equal loading and blotting. (B) HEK293 cells were exposed to 20% or 0.2% O_2 for up to 96 hours or to various etoposide concentrations up to 8 μM for one hour in normoxia. Phosphorylated and total H2AX were analyzed by immunoblotting.

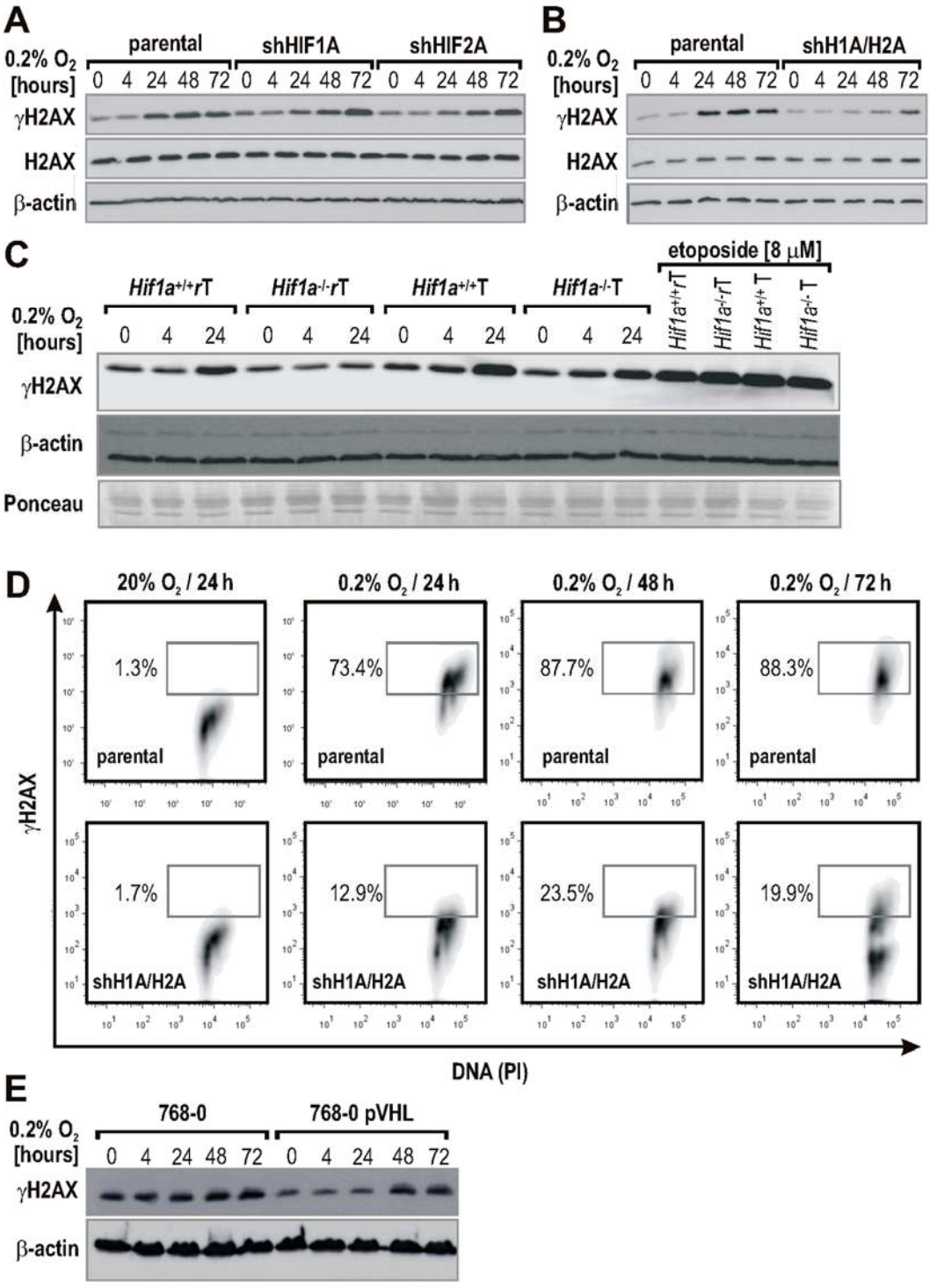

Figure 2 HIFs are required for hypoxic γH2AX accumulation.

(A, B) Parental, shRNA-mediated HIF-1α or HIF-2α knockdown (shHIF1A or shHIF2A, respectively) or HIF-1α/HIF-2α double knockdown (shH1A/H2A) HEK293 cells were grown under 20% or 0.2% O_2 conditions for the indicated time points. Phosphorylated and total H2AX was analyzed by immunblotting and β-actin served as a control for equal loading and

blotting. (C) MEF-*Hif1a*$^{+/+}$rT, MEF-*Hif1a*$^{-/-}$rT, MEF-*Hif1a*$^{+/+}$T, MEF-*Hif1a*$^{-/-}$T were exposed to 20% or 0.2% O_2 for 4 or 24 hours, or treated with 8 μM etoposide for 1 hour. Ponceau S staining was used as a control for equal extraction and loading of histones. (D) Parental and HIF-1α/HIF-2α double knockdown (shH1A/H2A) HEK293 cells were grown under 20% or 0.2% O_2 conditions for the time points indicated before γH2AX levels were analyzed by FACS. γH2AX positive cells were gated as indicated by the rectangles and quantified relative to the total cell number. (E) 768-0 and 786-0-pVHL cells were grown under 20% or 0.2 % O_2 conditions for 24 to 72 hours and γH2AX and β-actin protein levels were analyzed by immunoblotting.

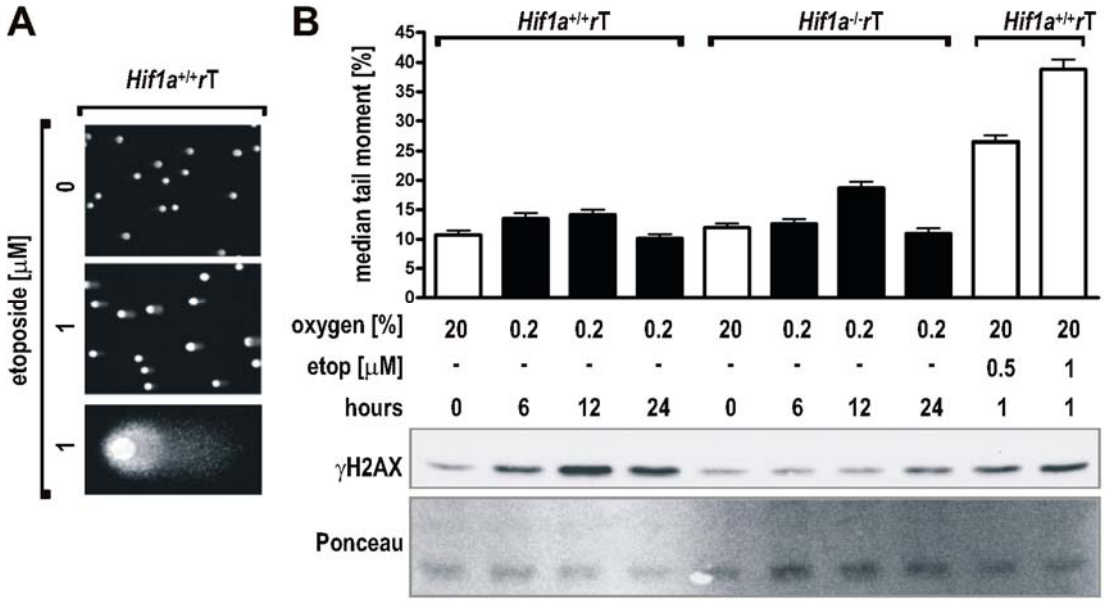

Figure 3 Hypoxia does not induce detectable DNA strand breaks.

(A) Representative example of a comet assay. DNA fragmentation in wildtype MEFs was induced by exposure to 1 μM etoposide for 1 hour. DNA was stained with SYBR green and all images were acquired with fixed exposure times. (B) DNA fragmentation was quantified by determining the median tail moment of at least 150 comets per condition using CometScore software. Data are shown was mean values ± SEM.